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TITLE: Ex Vivo Expanded (EVE) Megakaryocytes (MK) for Supportive
Care of Patients with Breast Cancer Hematologic
Malignancies: A Phase I/II Clinical Study

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The main goal of this project is to culture human hematopoietic stem cells in the presence of growth factors to produce autologous megakaryocytes (MK) to be transfused into patients as a supplement to the conventional stem cell transplant. The purpose of the clinical trial is to determine whether the transfused MK generate platelets in vivo in great enough numbers to reduce or eliminate the need for repeated platelet transfusions following high-dose chemotherapy and autologous stem cell transplant. Using the cocktail of growth factors thrombopoietin (from R&D), IL-3 and Flt3-L, the results obtained with our first patient are very encouraging with platelet recovery occurring at day 6 post-transplant (26,000/mm³). This unusual rapid recovery must be attributed to the *ex vivo* expanded cells. Midway into processing our MK cultures, we and other groups were notified by the FDA to avoid using thrombopoietin obtained from R&D because of concerns regarding the purification process. This new obstacle caused us to abort the transplant of *ex vivo* expanded MK for the second patient. We were fortunate to find an alternative form of clinical grade of thrombopoietin from Pharmacia which was approved by the FDA. We will reopen the clinical trial in two weeks. Despite the delays, we anticipate completing our project within the period of time covered by the no-cost extension of our grant.

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INTRODUCTION: The main goal of this project is to culture blood stem cells in the presence of human hematopoietic growth factors to produce autologous megakaryocytes (MK) to be transfused into patients as a supplement to the conventional stem cell autograft. The purpose of the clinical trial is to determine whether the transfused MK will generate platelets *in vivo* in great enough numbers to reduce or eliminate the need for repeated platelet transfusions following high-dose chemotherapy and autologous stem cell transplant.

As described in our last report (year 03), changes in the pharmaceutical industry have affected the supply of clinical grade growth factors causing us to revise our procedures on more than one occasion. Our initial grant proposal was based on growth factors provided by Searle, which was acquired by Pharmacia, terminating their growth factor development program. With considerable effort, we were able to generate similar preclinical data using a growth factor combination obtained from a variety of sources including Immunex (Flt3L) and R & D (thrombopoietin and IL-3). Our study was revised, an IND submitted and approved by the FDA, and our first patient entered. The results obtained with the first patient were very encouraging with full platelet recovery occurring at day 6 post-transplant. This unusual rapid recovery must be attributed to the *ex vivo* expanded cells. Midway into processing the expanded megakaryocytes for our second patient, we were notified that the FDA was no longer permitting the use of the thrombopoietin obtained from R & D, because of concerns regarding the purification process. This was a new obstacle that caused us to abort the cultures for the second patient. This new concern on the part of the FDA has affected research in cellular therapy at many centers.

We have been very fortunate to find an alternative source of TPO. Pharmacia has agreed to provide us with clinical grade thrombopoietin for which we have now obtained approval from the FDA. Our Office of Research and Sponsored Projects has been working on a contract that will have the approval of Pharmacia as well as Immunex (providers of the Flt3L). Whereas Amgen acquired Immunex at the very moment that we were seeking this approval, we have been delayed by their reorganization. Although verbal approval to proceed has been obtained, the final contract is pending, and is likely to take another week to complete. We anticipate reopening the trial within the next two weeks. There appear to be a large number of potential candidates who will be screened once the study is officially reopened.

We anticipate completing our project within the period covered by the no-cost extension of our grant.

BODY:

Materials and Methods:

Equipment

- 37° water bath, Scientific Products

- Class B sterile tissue culture hood, Baker Co.
- Clinical bench centrifuge, Beckman.
- 37° tissue culture incubator, 5% CO₂, Forma Scientific.
- Variable adjustable electronic pipettor, EDP, Rainin.
- Vacuum-driven automatic pipet device, Drummond.
- Coulter Epics XL 3-color laser flow cytometer, BeckmanCoulter Corp.

Materials

- Sterile pipet tips with aerosol barrier.
- Sterile individually wrapped polypropylene transfer pipets.
- 2ml, 5ml, 10ml, 25ml sterile individually wrapped polystyrene pipets with aerosol barrier.
- Sterile centrifuge tubes, Corning.
- 1cc, 3cc, 10cc, 20cc, 30cc, and 60cc sterile syringes, Becton Dickinson.
- 18G blunt nose needles, Becton Dickinson.
- Teflon tissue culture bags, 1L, (270ml nominal capacity) and 7ml capacity, Fluoroseal.
- Plastic volume-limiting pinch clips.
- Sterile Luer-Lok Connector tubing and bottle top septums, Nalgene.

Reagents

- Culture Medium: Sterile GMP-grade X-Vivo 20 serum-free medium, BioWhittaker, Walkersville, MD
- Heparin: Preservative-free, Life Technologies.
- DNase I: Roche Diagnostics. Reconstituted with sterile water and filtered through a
- Thawing Medium: X-Vivo 20 supplemented with 10 IU/ml heparin and 10µg/ml DNase.
- rhuTPO, GMP grade, R&D Systems, Minneapolis, MN. 250 µg per vial, reconstituted with 250µl sterile PBS
- rhuIL-3, GMP grade, R&D Systems, Minneapolis, MN. 25 µg per vial, reconstituted with 250µl sterile PBS
- Flt-3L, GMP grade, Immunex Corp, Seattle WA. 250 µg per vial, reconstituted with 250µl sterile water
20µL aliquots of each cytokine stored at -70° in sterile tubes until needed. Thawed aliquots of cytokine were kept at 4° for up to two weeks.
- 1% Trypan Blue (Sigma) in saline.
- Phycoerythrin-cyanin 5.1 (PC5) conjugated-α-CD34, Clone 581, Beckman Coulter,
- Phycoerythrin (PE) conjugated -α-CD41 Clone P2, Beckman Coulter
- Fluorescein isothiocyanate (FITC) conjugated-α-CD15, Clone 80H5, Beckman Coulter
- Clonogenic assay kits for CFU-MK (MegaCult C) and CFU-GM (MethoCult), Stem Cell Technologies, Vancouver, BC, Canada
- Quantikine huTPO ELISA, R&D Systems

Specimen Requirement

- Cells from a ten liter leukapheresis to be selected for CD34+ cells using the Clinimacs system. The CD34+ purified cells were then frozen by the Bone Marrow Transplant laboratory using the standard cell freezing system.

Procedure

A. Sample Preparation

1. Frozen CD34+ cell tube placed into a resealable plastic bag. Close bag (to prevent any water seepage into tube).
2. The bag with the tube is then placed in a 37° water bath and gently agitated until the cell suspension has thawed to a small ball of ice (approximately 3-4 minutes per 5 ml tube).
3. The bag is wiped dry and transferred to a Class B sterile tissue culture hood.
4. In the sterile tissue culture hood, the contents of each tube of thawed cells is transferred to a sterile 50ml centrifuge tube using a sterile polypropylene transfer pipet and diluted 1:10 by drop-wise addition of cold thawing medium using a sterile polypropylene transfer pipet with very gentle agitation/vortexing.
5. The tube is sealed and the cell suspension is centrifuged at 260xg for 10 min, 4°.
6. The cell pellet is resuspended in 2-3 ml X-Vivo 20 using a sterile polypropylene transfer pipet, then diluted to 50ml with X-Vivo 20 by decanting fresh medium. A 50µl sample for cell concentration analysis is taken using a sterile pipet tip with aerosol barrier. This sample is diluted 1:1 with Trypan Blue, applied to a hemacytometer, and the cell concentration and viability determined using a phase contrast microscope.
7. The cell suspension is centrifuged again at 260xg for 10 min at 4°. The cell pellet is resuspended in 2-3ml of X-Vivo 20 using a sterile polypropylene transfer pipet, then diluted with up to 20ml X-Vivo 20 by decanting fresh medium.

B. Culture Preparation

1. The final volume (FV) required to achieve a cell concentration of 400,000 cells/ml is calculated using the cell count obtained above in A.6.
2. The bag's nominal volume is adjusted with bag clip barriers so as to contain a volume sufficient to suspend the cells at 400,000 c/ml at an average fluid height of 1cm.
3. The cells are injected using a 20cc syringe into a 1L Teflon bag attached via Luer-Lok connections.
4. X-Vivo 20 and sufficient cytokines to achieve a final concentration of 100ng/ml Flt-3L, 100ng/ml TPO and 10ng/ml IL-3 are then added using a 60cc syringe attached via Luer-Lok connections to the bag to reach the FV calculated in B.1.
5. After gently agitating the bag to resuspend the cells, a 3ml aliquot is removed using a 10cc syringe. This is injected into a 7ml Teflon bag, adjusted with bag clip barriers so as to contain a volume sufficient to suspend the cells at an average fluid height of 1cm. This test sample is used to assay the culture progress and avoid opening the main sample bag until adjustment is necessary (See Sample Assays below). Both bags are stored in a 37° tissue culture incubator at 5% CO₂ and >90% relative humidity used solely for the purpose of clinical patient sample incubation.
6. Each day a 100µl of culture is removed from the test sample bag using a 1 ml syringe for cell concentration measurement. If the cell concentration is in excess of 800,000 c/ml, then a new bag clip barrier is placed on the bag so as to contain the new nominal volume required to dilute the cells to 400,000 cell/ml, and the old bag clip barrier is removed. The required volume of fresh X-Vivo 20 w/ cytokine cocktail is added to the main sample using a 60cc sterile syringe attached via Luer-Lok connections to the bag. If the new total volume exceeds the 1cm liquid height capacity of the bag, a new bag is attached to the old bag via a Luer-Lok connector tube, and the sample is equally divided between the two bags by suspending the bags until the volumes are equilibrated by gravity. Bag clips are used to keep the volume height at the specified 1cm.

C. Sample Assays

The following assays are performed at the start and end of the culture: Samples are washed and stained for phenotypes CD34, CD41, and CD15 and analyzed by flow cytometry.

1. 100,000 cell aliquots are washed in 1% BSA (Sigma) in PBS (GIBCO) with 5mM EDTA (Sigma), designed to prevent further platelet activation and/or reverse adherence of activated platelets. The wash buffer is aspirated and the pellet resuspended in 50µl of PBS.
2. After washing, the cells are stained for 15 min at 4°C in the dark with 5µl of PC5-CD34, PE-α-CD41, and FITC-α-CD15 and analyzed by flow cytometry on the same day. The negative controls are PC5-, PE- and FITC-α-mouse IgG₁ used at equivalent IgG₁ concentrations. Only the non-apoptotic high forward scatter, low side scatter cell population was used for subset analysis.
3. Clonogenic assays are performed according to the manufacturer's instructions. Viable cells from each day of assay are seeded at a concentration of 10³ cells/ml. CFU-MK are scored after ten days and CFU-GM/BFU-E after fourteen days.
4. Twenty-four to forty-eight hours prior to the culture endpoint, a small aliquot of the patient cell culture is assayed for the following: Quantitative PCR for malignancies other than breast cancer and histochemistry for breast cancer cells. Sterility: Bacterial, viral, and fungal contamination, endotoxin titer, and mycoplasma levels.
5. When the designated culture period is reached, the sample bag(s) is(are) attached via Luer-Lok connector tubes to a cell washing bag and transfer to a COBE 2991 Cell Washer. Cells are prepared for transplant according to Bone Marrow Transplant Laboratory procedures.
6. A small aliquot of the buffer from each round of washed cells is assayed for residual TPO by ELISA, following manufacturers instructions, to monitor effectiveness of washing at removing cytokines from cell suspension.

Results:

Patient 1: Culture of the CD34+ cells was started at day -6 before transplant. Cells expanded significantly (table 1). On day 0 (day 6 of culture), the conventional autograft consisting of 6.55×10^6 CD 34+ cells/kg was infused, followed two hours later by the expanded product, which after washing contained a total of 8.3×10^6 total cells/kg, 20×10^4 CFU-GM/kg, 16×10^4 CFU-MK/kg, and 1.9×10^6 CD41+ cells/kg.

Table 1. EX VIVO EXPANSION RESULTS

	Total Cell No. ($\times 10^8$)	Viability (%)	Number of Progenitors per CD34 ⁺ Cell Seeded*				
			CD34 ⁺ /41 ⁺ ($\times 10^6$)	CD41 ⁺ ($\times 10^6$)	CFU-MK ($\times 10^4$)	CFU-GM ($\times 10^4$)	BFU-E ($\times 10^4$)
Day 0	1.04	82	5.4	5.4	1.02	1.22	2.3
Day 6	7.95	70	11.4	13	5.10	6.50	7.5
Total Infused (per kg)			1.6	1.9	16.0	20.0	23.0

*CD34⁺ cells expanded 4.3-fold during the 6 days in culture

Cultures for day -2 (bacterial, fungal, and gram stain) were negative, as were cultures and gram stain from the day of infusion. Endotoxin assay was negative (< 0.25 EU/kg patient weight), and the mycoplasma cultures performed by Safe Cells testing laboratory were also negative. Specimens will be batched to assay for residual TPO in the future (0.8 ng/ml, n=6, in preclinical studies). There was no toxicity associated with infusion of the *ex vivo* expanded megakaryocytes.

Toxicity of the high-dose chemotherapy and autologous stem cell transplant through hospital discharge was notable for grade 3 febrile neutropenia occurring on day +5 post-transplant, grade 3 fatigue, and grade 3 hyponatremia occurring on day -2, related to hydration and high-dose cyclophosphamide. As expected in an autologous stem cell transplant, the patient had grade four neutropenia, leukopenia, and thrombocytopenia.

The patient received only two platelet transfusions. She first became thrombocytopenic (platelets $< 20,000/\text{mm}^3$) on day +5, with $5,000/\text{mm}^3$ (Fig. 1) and was given a single donor plateletpheresis pack. The following day, day +6, her platelets were 26,000, but in error, she was again transfused platelets. Subsequently, she never dropped her platelet level below $20,000/\text{mm}^3$, and by day 10 post-transplant her platelets were $53,000/\text{mm}^3$. By day 11, her platelets were $100,000/\text{mm}^3$, peaking at $322,000/\text{mm}^3$ on day +16 (Fig.1).

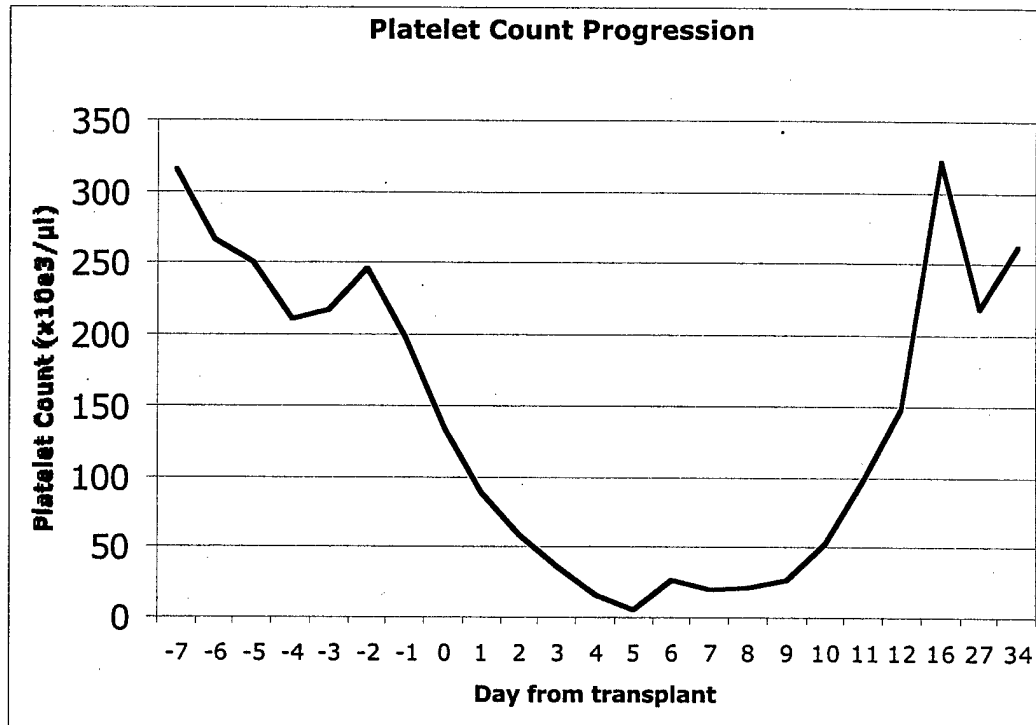


Fig. 1

Her platelets have subsequently remained above 200,000/mm³. This rapid recovery to very high platelet numbers is distinctly unusual for an autotransplant patient and must be attributed to the *ex vivo* expanded cells. Platelet function testing performed at the time of recovery were normal.

Patient 2: This patient underwent apheresis without complication. Cells were CD34+ selected and placed in culture. A clinical "HOLD" was placed on the protocol March 1, 2002, because issues had been raised by the FDA regarding the Thrombopoietin (see INTRODUCTION).

KEY RESEARCH ACCOMPLISHMENT: Successful platelet engraftment of our first patient.

CONCLUSIONS: The results from the first patient were positive and encouraging. Total cells expanded almost 8-fold after 6 days of culture, were significantly viable, no negative reactions were observed, and the patient recovered normal platelet levels faster than historical controls, with normal platelet function. The rapid platelet recovery is unusual and must be attributed to the *ex vivo* expanded cells. We had to abort transplanting the second patient as the FDA no longer permits the use of the thrombopoietin obtained from R & D, because of concerns regarding the purification process. This new concern on the part of the FDA has affected research in cellular therapy at many centers. Pharmacia will provide us now with clinical grade thrombopoietin for which we have now obtained approval from the FDA. We anticipate

reopening the trial within the next two weeks. We have a large number of potential candidates who will be screened once the study is officially reopened. Despite the delay we anticipate completing our project within the period covered by the no-cost extension of our grant.